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# "Molecular cophylogeny of lagriid beetles and their endosymbiotic bacteria"

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# II. ABBREVATIONS

| | |
|---|---|
| Bact. | Bacteriome |
| BSM | Bootstrap Method |
| CFU | colony forming unit |
| cond. | condition |
| div. | diverse |
| *Ecno.* | *Ecnolagria sp.* |
| *EDTA* | Ethylenediaminetetraacetic acid |
| EtOH | Ethanol |
| Fig. | figure |
| HB | Hybridisation Buffer |
| HCL | Hydrogen chloride |
| Ident. | Identity |
| *L. ruf* | *Lagria rufipennis* |
| *L. nig* | *Lagria nigricollis* |
| *Macro*. | *Macrolagria* |
| Mag | Magnification |
| min | minute |
| ML | Maximum likelihood |
| NNI | Nearest-Neighbor-Interchange |
| No. | number |
| Rep. Sys | reproductive system |
| rpm | revolutions per minute |
| s | seconds |
| SDS | Sodium dodecyl sulfate |
| *sp*. | species (for epithet) |
| $T_a$ | annealing temperature |
| TRIS | Tris(hydroxymethyl)-aminomethan |
| WB | washing buffer |

# 1. ZUSAMMENFASSUNG/SUMMARY

Die vorliegende Arbeit ist eine experimentelle Betrachtung von Lagriiden (Coleoptera) – *Burkholderia* (beta-Proteobakterien) - Symbiose. Hierbei stellen die 7 (später insg. 9) verschiedenen geographisch weit verbreiteten Lagriiden-Arten die potentiellen Wirte für die jeweiligen extrazellulären, in erwarteten Bakteriensäckchen beherbergten Endomikroben dar. Proben aus Japan, Australien, Südafrika, Brasilien und Mitteleuropa sind verfügbar. Es wird das Vorhandensein dieser Organe und die Identität der Bakterien mittels molekularer Analyse von verschiedenen Genen (Rekombinase A, Gyrase B und der bakteriellen 16S rRNA) in den bereitgestellten Proben festgestellt und die jeweiligen phylogenetischen Verwandt-schaftsverhältnisse von Wirten (anhand der Cytochrom Oxidase I, 18S und 28S rRNA Gene) und Symbionten getrennt rekonstruiert. Um eine mögliche, durch enge symbiontische Assoziation hervorgerufene Co-Speziation von Wirten und Symbionten festzustellen, werden die erstellten Stammbäume miteinander verglichen um übereinstimmende oder voneinander abweichende Artaufspaltungsereignisse nachzuvollziehen. Für die Analyse wurden die Insekten seziert, die jeweiligen Organe und Gewebe entnommen, die DNA isoliert, per PCR die genannten Gene mit den passenden Primern amplifiziert und die gewonnenen Fragmente sequenziert. Die Software Geneious 5.0.6 und BLAST werden verwendet um eine Stammbaumerstellung mit den Programmen MEGA 5 und FastTree 2.1.7 zu ermöglichen. Eine Floureszenz-in-situ-Hybridisierung (FISH) wurde zur optischen Darstellung der betreffenden Organe des Experiments angefertigt. Die Ergebnisse der morphologischen Untersuchung und Genfragment-Analysen zeigen, dass insgesamt 4 Lagriiden Arten die Symbiose per Bakteriensäckchen ausbilden. Die Identität der enthaltenen Organismen ist in allen untersuchten Fällen soweit die Bestimmung möglich war vermutlich *Burkholderia gladioli,* jedoch ist die Bestimmung der jeweiligen Stämme nicht eindeutig. Zur phylogenetischen Rekonstruktion wurden letztendlich nur die Gene der 16S, 18S und 28S rRNA herangezogen, da sie qualitative hochwertige Sequenzen in brauchbarem Probenumfang lieferten. Die Endobakterien durchmischten sich in der Phylogenie mit nicht-symbiontischen Bakterien der verschiedenen *Burkholderia gladioli*

Stämme. Dies weist auf einen zu Teilen beteiligten horizontalen Transfer der Symbionten hin. Bisher war durch Beobachtungen an der Art *Lagria hirta* bekannnt, dass durch die mütterliche Linie eine vertikale Weitergabe der Bakterien an das Gelege der Fall ist. Zukünftig liegt die Rekonstruktion aussagekräftiger Phylogenien durch Betrachtung von mehr Genen, sowie die Fragen nach der Funktion der endobakteriellen Gattung *Burkholderia* für die Lagriid Familie, Faktoren der Symbiose-Etablierung und die ökologischen Gründe, welche hohe Spezifität und die weite geographische Verbreitung der Assoziation vermitteln, im Interesse der wissenschaftlichen Betrachtung.

The present thesis is an experimental approach to the Lagriid (Coleoptera) – *Burkholderia* (beta-proteobacteria) - symbiosis. In this case the 7 (9 at the late progress) different geographically distributed Lagriid species function as potential hosts for the particular extracellular endomicrobes expected to be harboured in bacteriomes. Samples from Japan, Australia, South-Africa, Brasil and Middle Europe are available. The presence of the organs and the bacterial identity is determined in the provided samples by morphological examination and molecular analysis of different genes (Recombinase A, Gyrase B and the eubacterial 16S rRNA). The particular phylogenetic relationships between hosts (on the basis of Cytochrome Oxidase I, 18S und 28S rRNA genes) and seperately between the bacteria will be reconstructed. To find a possible co-speciation caused by intimate symbiotic association of hosts and their symbionts, the resulting trees are compared in order to detect congruent or deviating events of speciation. For the analysis the insects were dissected, the organs and tissues of interest extracted, the DNA isolated, the genes amplified via PCR and the gained fragments were sequenced. By the software Geneious 5.0.6 and the tool BLAST tree reconstruction with the programs MEGA 5 und FastTree 2.1.7 was fascilitated. A flourescence-in-situ-hybridization (FISH) was applied to visualize the organs of experimental focus. The results of morphological observation and gene fragment-analysis show that 4 Lagriid species develop the symbiosis via bacteriomes. The identity of all examined cases - as identification was possible - showed presence of *Burkholderia gladioli,* but with insecurity in strain determination. Finally for phylogenetic reconstruction only the genes of 16S, 18S und 28S rRNA were used because they delivered useful yields of high qualitaty sequences. The endobacteria

intermingled with non-symbiotic bacteria. So far it was observed that *Lagria hirta* is infecting the offspring by eggsmearing, followed by bacterial immigration through the eggshell. The present results suggests an occasional horizontal transfer combined with a vertical transmission mode of symbiosis maintainance from one generation to the next. In possible future research stable phylogenetic reconstruction based on more genes same as clearifying the function of the endobacteria of the genus *Burkholderia* for the Lagriid family, factors for symbiosis establishment and the ecological reasons mediating the high specifity and broad geographical distribution of the association between Lagriids and *Burkholderia gladioli* will be the main questions of interest.

# 2.INTRODUCTION

Symbiosis is a common strategy among living entities, connecting two different organisms as symbiont and host in a long-term and in certain cases strongly dependent relationship. The influences on physiological or ecological traits for both can range from beneficial (mutualism) to neutral (commensalism) or even prasitic interdependencies. Endosymbiosis is a very intimate partnership and can provide a variety of effects. Genomic reduction in microbial symbionts can be a consequence of this intimacy and is correlated with obligate and intracellular endosymbiosis. Extracellular endosymbionts are equally or possibly more prevalent, and are observed every day more often in symbiosis research [1, 2]. The current study will give insight on an insect-microbe interaction occurring between Coleoptera and extracelluar bacterial endosymbionts harboured in structures associated to the ovipositor. The results reveal the identity and phylogenetic associations of the bacterial symbionts, which possibly reflect common patterns when compared to the the host's evolutionary lineage. Thereby, this project gives first steps in elucidation of an hypothesis on how the symbiosis is maintained, when the symbiosis was established, whether it is present only in a monophyletic clade of Lagriids and if coevolution between the symbiotic partners has occurred.

## 2.1 ECOLOGY OF SYMBIOSIS

Mutualism is defined as bidirectional benefit for symbiont and host. In case of microbes as partners the physiological and ecological role does not nescessarily have to be elucidated in order to call them symbionts. It could also be a commensalistic or even parasitic relationship, but still the host provides the habitat for the microbe. Interdependencies mediate different varieties of exchanged properties and influences on ecological traits such as defense by toxins, nutritional benefits that can be microbe mediated accession to compounds or receiving metabolic intermediates the host is lacking in its own biosynthetic pathways or conferring the ability to live in otherwise unbearable environments (e.g. heat-tolerance) [3]. Regarding defense, bioactive compounds can be provided, such as polyketides known for antimicrobial [1] or antifungal effects. [4] Ways of symbiosis develop in a gradient manner from facultative to obligate while the obligate and very intimate connection increases the possibility of co-cladogenesis between host and symbiont. [5] Also it is observed that nutritional benefits to the host are associated with long-term codiversification [1]. Codiversification means a connected radiation of the symbiont species to its host species radiation. Closely and intimate associations normally involve vertical transmission of the microbe to the next generation via the maternal line [3]. In the facultative case microbes can often be cultured, while obligate symbionts usually do not survive outside the host organism. Most likely the host also suffers strongly in the absence of the symbiont, which can lead to lethality depending on the strength and intimacy of the relationship [5].

## 2.2 THE LAGRIINAE – *BURKHOLDERIA* SYMBIOSIS

*Lagria hirta*, a species belonging to the Lagriinae subfamily, carries a bacterial endosymbiont of the species *Burkholderia gladioli* in specific enclosed structures located dorsally in larvae and in a pair of organs connected to the ovipositor in adult females. Males possibly lose their endosymbionts during metamorphosis by an unknown mechanism or never acquire the endosymbionts [6].

The bacterial symbionts are harboured in gland-like structures which were named intersegmental sacs ("Intersegmentaltaschen") and ovipositor sacs ("Legeapparattaschen") [7]. Along the presented experiment the focus is mainly on the intersegmental sacs, which are referred to as bacteriomes. These organs are structures of fine transparent tissue located inside the abdomen connected laterally to the reproductive system and filled with extracellular bacteria. As the eggs pass through the ovipositor the bacteria-enriched substance covers their surface and hence the position of these organs has been assumed to mediate the vertical transfer of the microbe association via egg smearing. As it was observed so far, there are no bacteriomes found in the male specimen, but there occurs a variety of possible morphological shapes in different Lagriid species. They differ from simple sacks to elongated and even branched tubes or burred and cleaved structures [7].

## 2.3 The Lagriinae

The lagriinae subfamily belongs to the darkling beetles – (Tenebrionidae, Coleoptera) *Latreille 1802* - and is characterized by a hard body, carrying hairy elytra, the tarsal formula 5-5-4 and holometabolic ontogenesis. The members of the family are primarily saprophagous and usually feed on a variety of dead plant material [8]. The Tenebrionidae are the largest lineage of the superfamily Tenebrionoidea with approximately 19 000 species in more than 2000 genera worldwide. However, the phylogenetic relationships within the Tenebrionidae remain unclear. Tenebrionoidea (formerly Heteromera, *Latreille 1803*) is a species rich, morphologically and ecologically heterogenous superfamily of polyphagan beetles and has been accepted as a lineage within Cucujiformia [8]. Coleopterans in general have the greatest distribution and variety of species of all insect taxa. The habitats, feeding and associations with other organsims cover a broad range of possibilities. Thus, the ecological role and identity of recent species is overwhealmingly great and in most cases unknown.

## 2.4 The Genus *Burkholderia*

The abundant genus *Burkholderia* (class Betaproteobacteria, order Burkholderiales) consists of aerobic gram-negative rod-shaped bacteria who are often associated with plants, fungi, soil and insects. They can have beneficial, parasitic, pathogenic or neutral effects on hosts as symbiotic partner or even pathogenic effects on humans [9]. More than 60 species are included in the genus and split in two main phylogenetically defined groups as Estrada-de los Santos et al. [10] stated based on multilocus sequence analysis. Group A includes plant associated and saprophytic species who represent many of the diazotrophic species beneficial to plant nodulation as *Burkholderia phymatum* in the legume *Phaseolus vulgaris* and *Mimosa* [11] while group B contains pathogenic species (human and plant pathogens). *Burkholderia gladioli,* formerly known as *Pseudomonas marginata* belongs to this group and is originally known to cause soft rot in onions, as well as iris and gladiolus plants [12]. *Burkholderia* species were included in the *Pseudomonas* genus before research on 16S rDNA showed significant similarities among the species of the now distinctly designated genus *Burkholderia*. The species of the genus are found as microbial endosymbionts in fungi for example as a putative nitrogen fixing unit of the arbuscular mycorrhizal fungus *Gigaspora margarita* [13] or *Burkholderia rhizoxinica* as an intracellular symbiont of the phytopathogenic fungus *Rhizopus microsporus* who additionally supports the host's sporulation capability [14]. A well studied case is the symbiosis between stinkbugs (e.g. *Riptortus*) and *Burkholderia* species as extracellular symbionts harboured in specialized midgut crypts providing insecticide and stress resistance [15].

## 2.5 Experimental design

Different molecular markers, along with fluorescent-in-situ-hybridization, in order to investigate the prevalence and identity of endosymbiotic bacteria in lagriinae beetles were used in this experiment. The present host sample collection consists of the species *Lagria rufipennis, Lagria nigricollis, Macrolagria rufobrunnea, Arthromacra viridissima, Ecnolagria sp. (grandis?), Casnoidea dimidiata,*

*Lagria sp.* and provided data of *Lagria hirta* and *Lagria villosa*. Fig.1 shows their taxonomic classification. Furthermore, potential host-symbiont cocladogenesis has been assessed by reconstructing and comparing the phylogenetic relationships of each group based on three different molecular markers for insects and three for bacteria. The comparison of the bacterial symbiont and host lineage pattern showing the paths of diversification of each partner gives insight into the specificity of the associations and reflects the mode of transmission which can range from strictly vertical, vertical with occasional horizontal transfer to only horizontal.

# 3. Material and Methods

## 3.1 Samples

Order: Coleoptera – Polyphaga – Cucujiformia

Superfamily: Tenebrionoidae

Family: Tenebrionidae – LATREILLE, 1825

Subfamily: Lagriinae LATREILLE, 1825

- ***Lagria hirta* LINNAEUS , 1758**
- ***Lagria rufipennis* MARS.**
- ***Lagria nigricollis* HOPE, 1843**
- ***Lagria villosa* FABRICIUS**
- ***Arthromacra sp. (viridissima)* KIRBY 1837**
- ***Ecnolagria sp. (grandis)* GYLLENHALL, 1807**
- ***Casnoidea dimidiata* FAIRMAIRE, 1887**

Tenebrionidae incerta sedis

- *Macrolagria sp. (rufobrunnea)*

Fig. 1: Taxonomy of the closely related Lagriid species used for experimental purpose, (underlined) [16, 16a , 17]

## 3.2 Dissection

The samples were dissected to separate the tissues of interest such as bacteriomes and host body. The collected samples and all of their characteristics including origin and condition are displayed in table 1. The process of dissection was carried out in Ethanol in a wax filled petri dish. The field-collected insect samples were stored in different fixatives, which influenced the conditions in a different

manner. Either RNA*later*, Ethanol or Acetone were chosen by the particular collector. A few samples carried fungal contamination on their surface (table1). To fix the insect on the wax a pin was used to pierce the prothorax through its covering scutum from dorsal so it was easy to cut the elytron and wings at their base. Next was cutting lateral along the pleurit at least half way up the abdomen from between the two cerci at the posterior segment of the abdomen on. Now the dorsal chitinous exoskeleton could be flipped so the internal organs such as gut, ovaries, reproductive system and the malpighian tubule system were visible. The main parts of interest were the bacteria storing structures. This structure was isolated carefully while the ovipositor and the bacteriomes were separated. The bacteriomes, reproductive system and rest of the body were separately stored in screw top Eppendorf tubes containing 95% Ethanol.

### 3.3 DNA Isolation

The tissue samples displayed in table 1 were treated with an Epicentre Master Pure™ DNA Isolation Kit as follows: To homogenize the sample liquid nitrogen was added to the sample before grinding it. 300 µl of Tissue and Cell Lysis Solution and 5 µl of ProK (Protein Kinase, 10 mg/ml) were added before an incubation period of 15 min at 60 – 65°C. Afterwards the samples were placed on ice for 3 to 5 min to cool down before adding 150 µl of MPC Protein Precipitation Reagent. After vortexing and 10 min of centrifugation (10000 rpm) a pellet is formed. The supernatant was transferred and the pellet discarded, so 500 µl of isopropanol were added into the DNA containing supernatant. After inverting and 15 min storage at -20°C a pellet was recoverd by repeated centrifuging at 14000 rpm for 10 min. The extracted pellet was washed with 200 ml of cold 70% Ethanol and centrifuged again (5 min, 14000 rpm). The supernatant was discarded and the sample left to dry out under the fume hood. Resuspension was carried out in 100 µl of Low TE , half of each sample was then stored in an extra tube -80°C, while the other half of it was kept in -20°C for short-term use.

Table 1: List of dissected species and organs

| | Species | Sex | Origin & Storage | dissected organs | notes |
|---|---|---|---|---|---|
| **1.1** | *Lagria rufipennis* | female | Japan,RNA-later | Bacteriome, Reproductive System, Body, Wings,Legs&Head | condition very dry |
| **1.2** | *Lagria rufipennis* | female | Japan, Ethanol | Bacteriome, Reproductive System, Body, Wings,Legs&Head | condition very dry |
| **1.3** | *Lagria rufipennis* | female | Japan, Ethanol | Bacteriome, Reproductive System, Body, Wings,Legs&Head | condition very soft |
| **1.4** | *Lagria rufipennis* | female | Japan, Acetone | Bacteriome, Reproductive System, Body, Wings,Legs&Head | ripped gut |
| **2.1** | *Lagria nigricollis* | female | Japan, RNA-later | Bacteriome, Tubes, Rep.Sys., Body, Wings,Legs&Head | ripped gut,low amount of fungus |
| **2.2** | *Lagria nigricollis* | female | Japan, RNA-later | Bacteriome, Reproductive System, Body, Wings,Legs&Head | low amount of fungus |
| **2.3** | *Lagria nigricollis* | female | Japan, RNA-later | Bacteriome, Tubes, Rep.Sys., Eggs, Body, Wings,Legs&Head | in very good condition |
| **2.4** | *Lagria nigricollis* | female | Japan | Bacteriome, Reproductive System, Body, Wings,Legs&Head | |
| **3.1** | *Macrolagria rufobrunnea* | female | Acetone | Reproductive System, Body, Wings,Legs&Head | no visible Bact.;condition very good |
| **4.1** | *Arthromacra viridissima* | female | Japan, Acetone | Bacteriome(?), Reproductive System, Body, Wings, Legs &Head | Bact. not identified; cond. good |
| **5.1** | *Ecnolagria spec. (grandis?)* | female | Australia, Ethanol | Bacteriome, Reproductive System, Body, Wings,Legs&Head | elongated Bact. structures |
| **6.1** | *Casnoidea dimidiata* | male | Australia, Ethanol | Reproductive System, Body, Wings,Legs&Head | In very good condition |
| **6.2** | *Casnoidea dimidiata* | male | Australia, Ethanol | Reproductive System, Body, Wings,Legs&Head | In very good condition |
| **7.1** | *Lagria spec.* | male | South Africa | Reproductive System, Body, Wings,Legs&Head | In very good condition |
| **8.1** | *Lagria hirta* | female | Germany | | Not dissected |
| **9.1** | *Lagria villosa* | female | Brasil | | Not dissected |

## 3.4 PCR

The used genes for phylogenetic reconstruction, the corresponding primers and information about the amplification conditions are listed in table 2. The forward primer burk16S1F and the reverse primer burk16S1R were used to obtain information about the actual presence of *Burkholderia* species in the samples.
12,5 µl is the standard PCR reaction volume in this experiment and contains 1,25 µl Buffer, 0,25 µl of 2,5 mM MgCl2, 1,5 µl dNTPs of 2 mM concentration, 6,5 µl H2O (Millipore), 1 µl of 1:10 diluted forward and reverse primer, which had a concentration of 10 pmol and then 0,5 U/µl Taq-Polymerase. 1 µl of DNA suspension is added to each PCR reaction. The whole process of mixing the recipe is done on ice. For the host PCRs firebug (*Pyrrhocoris apterus*) DNA was used as a positive control. A watersample was used as a negative control instead of 1 µl template DNA in order to control for possible contamination.

The 18S rRNA gene fragment is of about 2000 bp in length. To solve the problem of too long fragments, a primerwalk was used to amplify the chosen sequence in several steps. Therefore eight primers were taken to achieve a length of approximately 500-700 bp with each primerpair. The resulting sequences would be consecutively assembled in the program Geneious 6.0 by creating a consensus sequence of all aligned overlapping short fragments, so at the end the result would be the 2000 bp sequence.

## 3.5 Gel electrophoresis

PCR products were visualized using gel electrophoresis. In each case a 1,5% Agarose-Gel (0,75 mg Lonza, SeaKem® LE Agarose per 50 ml TBE Buffer) was prepared, then loaded with 5 µl GelRed® and ran in an OWL Easycast B1 Chamber at 150 A for 30 min. The first Slot contained the Gene Ruler while the following slots were filled with 1- 3 µl Green Buffer Loading Dye mixed with 3 – 5 µl of the particular sample. To visualize the fragments, a transilluminator (G:Box, Syngene) with UV light spectrum was used and pictures were recorded by GeneSnap software.

The cleaning of PCR products was conducted with an innuPREP PCR pure kit (Analytik Jena) which is a silica membrane column based cleaning kit. All components of the PCR reaction but the DNA Fragment should be washed out after the treatment. First step was to place a Spin Filter onto a Receiver Tube. This was filled with 500 µl of Binding Buffer and the PCR reaction mix was added before centrifuging the sample at 10000 x g for 2 min. The Receiver Tube is discarded and the Spin Filter placed on an Elution Tube. After 1 min incubation time with 30 µl of Elution Buffer the final centrifugation step of 1 min at 6000 x g is done to elute the cleaned PCR product.

After the colony PCR, the samples were extracted with a QIAquick® PCR Purification kit. First the Fragment was excised with a scalpel and the gel slices were weighted separately. 300 µl GQ solubilization buffer (contains Guanidine, Thiocyanate and a pH-Indicator) was added per 100 mg gel and left for incubation on a heating block at 50°C for at least 10 min. After complete dissolving, 100 µl isopropanol for DNA precipitation was added per 100 mg of gel weight. A QIAquick spin column was placed in a 2 ml collection tube and filled with the sample. After 1 min in the centrifuge (13000 rpm), the flow through was discarded and 500 µl PB (binding) Buffer (contains Guanidine, hydrochloride, isopropanol) was added to the column and again centrifuged for 1 min. This was repeated after adding 750 µl of PE (wash) Buffer (contains Ethanol) and then again after discarding the flow through. The elution of the bound DNA from the silica membrane was conducted by adding 50 µl of EB (elution) Buffer (10 mM Tris-Cl, pH 8,5). After incubation of 1 min the final centrifugation step was performed to receive the extracted PCR product.

### 3.6 CLONING

Cloning was used to recover and amplify single 16S eubacterial sequences from the amplified eubacterial DNA. This was supposed to show the identity of the fragment's origin by the following step of sequencing. Fragments for cloning were initially obtained by amplifying the eubacterial 16S rDNA gene (1400 bp) as described previously for the PCR procedure on this gene. The vector used for the

transformation was 2.1 TOPO Vector and a TOPO TA Cloning ® kit was used as described in its mannual. The vector contained antibiotic resistency (Kanamycin, Ampicillin) to allow growth on selective media and consequently to show a successful transformation. Additionally the vector coded for a functional sequence of ß-Galactosidase (lac Z), which could be used for blue/white screening of the colonies. Only white CFUs were used for the following colony PCR and sequencing.

### 3.7 Colony PCR

The protocol of colony PCR is the same as described above for PCR. The difference consists of the DNA sample, which was included as a CFU instead of 1 µl DNA. The sample was added to the PCR reaction tubes by a toothpick right before starting the program MKMS-55. The forward primer was T7, the reverse primer M13.

### 3.8 Sequencing

The PCR products of each gene listed above were sequenced to analyze the fragments. Estimation by the semi-quantitative PCR method was done by sense of proportion of the brightness each particularly selected sample showed in the gel which was drawn by eye. For the brightest bands 1 µl was taken out of the cleaned PCR product, for half brightness 1,5 and for the very slight and pale bands 2 µl were used. The mixture for sequencing contained 1 µl of primer so every chosen sample was given to be sequenced together with the forward and in another reaction tube together with the matching reverse primer. The sequencing reaction mix was then filled up to 6 µl with Merck® water. The sequencing was conducted with a 3730XL, Terminator Cycle Sequencing Kit (Applied Biosystem).

### 3.9 Sequence analysis

The sequences were trimmed and corrected manually in the software program GENEIOUS 6.0.5 after the forward and reverse sequenced fragments were aligned to a reference sequence which was taken out of the database GenBank so the approximate distance between non-overlapping fragments from each sequencing orientation could be determined correctly. In case of overlapping regions, the homologous positions were compared and as a result a consensus sequence could be created after correcting low quality sequencing errors in the two sequences. In cases where no certain base could be found or a decision between two different bases could not be made for sure a degenerated replacement code was used: N – any base possible, R – Adenine or Guanine, M – Adenine or Cytosine, W – Adenine or Thymidine, Y – Thymidine or Cytosine, K – Thymidine or Guanine, S – Guanine or Cytosine. The resulting consensus sequence could be used to identify a certain species out of the database GenBank by the Basic local Alignment tool (BLAST).

Table 2: all Primers used for PCR reactions

| **Insect Primers** | | | | | | **PCR Program** |
|---|---|---|---|---|---|---|
| **Target** | **Primer** | **Sequence** | **Orientation** | **Target-Size** | **Reference** | |
| **18S coleoptera** | 18S 5´ | GACAACCTGGTTGATCCTGCCAGT | F | 500 | Shull 2001, Levkanikova 2009 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S b5.0 | TAACCGCAACAACTTTAAT | R | | Shull 2001, Levkanikova 2009 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S ai | CCTGAGAAACGGCTACCACATC | F | 500 | Shull 2001, Levkanikova 2009 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S b2.5 | TCTTTGGCAAATGCTTTCGC | R | | Shull 2001, Levkanikova 2009 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S a1.0 | GGTGAAATTCTTGGACCGTC | F | 500 | Shull 2001, Levkanikova 2009 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S bi | GAGTCTCGTTCGTTATCGGA | R | | Shull 2001, Levkanikova 2010 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S a2.0 | ATGGTTGCAAAGCTGAAAC | F | 500 | Shull 2001, Levkanikova 2011 | Coleo18S, 55°C $T_a$, 35 cycles |
| | 18S 3'I | CACCTACGGAAACCTTGTTACGAC | R | | Shull 2001, Levkanikova 2010 | Coleo18S, 55°C $T_a$, 35 cycles |
| **28S coleoptera** | 28Sff | TTACACACTCCTTAGCGGAT | F | 670-760 | Inward 2003, Levkanikova 2009, Gomez 2005 | coleo28S, 55°C $T_a$, 40 cycles |
| | 28Srr | GGGACCCGTCTTGAAACAC | R | | Inward 2003, Levkanikova 2009, Gomez 2006 | coleo28S55°C $T_a$, 40 cycles |
| **COI general** | Jerry | CAACATTTATTTTGATTTTTTGG | F | 723 | Simon et al 1994, Levkanikova 2009 | COI-50,50°C $T_a$, 39 cycles |
| | Pat | TCCATTGCACTAATCTGCCATATTA | R | | Simon et al 1994, Levkanikova 2009 | COI-50,50°C $T_a$, 39 cycles |
| **Bacterial Primers** | | | | | | |
| **16S Eubacteria** | fD1 | AGAGTTTGATCCTGGCTCAG | F | 1400 | Weisburg et al. 1991 | MKMS, 65°C $T_a$, 31 cycles |
| | rP2 | ACGGCTACCTTGTTACGACTT | R | | Weisburg et al. 1991 | MKMS, 65°C $T_a$, 31 cycles |
| **Cloned 16S Eubacteria** | T7 | TAATACGACTCACTATAGGG | F | 1400 | | MKMS, 55°C $T_a$, 31 cycles |
| | M13 | GGAAACAGCTATGACCATG | R | | | MKMS, 55°C $T_a$, 31 cycles |
| | R1087 | CTCTTGCGGGACTTAACCC | R | 800 | Criminal and environmental soil forensics, 2009 Ritz et. Al, Page 384 | no PCR, only sequenced |
| **16S Burkholderia** | Burk16S_1_F | GTTGGCCGATGGCTGATT | F | 200 | - | Burk1, 60°C $T_a$, 32 cycles |
| | Burk16S_1_R | AAGTGCTTTACAACCCGAAGG | R | | - | Burk1, 60°C $T_a$, 32 cycles |
| **recA Burkholderia** | recA-Burk_F | AGGACGATTCATGGAAGAWAGC | F | 704 | Spilker 2009 | RecA-Burk, 58°C $T_a$, 31 cycles |
| | recA-Burk_R | GACGCACYGAYGMRTAGAACTT | R | | Spilker 2009 | RecA-Burk, 58°C $T_a$, 31 cycles |
| **gyrB Burkholderia** | gyrB-Burk_F | ACCGGTCTGCAYCACCTCGT | F | 738 | Spilker 2009 | gyrB-Burk, 60°C $T_a$, 30 cycles |
| | gyrB-Burk_R | YTCGTTGWARCTGTCGTTCCACTGC | R | | Spilker 2009 | gyrB-Burk, 60°C $T_a$, 30 cycles |

## 3.10 Phylogenetic tree reconstruction

For the reconstruction of the phylogenetic relationships among the host samples (based on 28S, 18S Coleoptera rRNA gene sequences) and separately the associated bacteria (Eu 16S rRNA, gyrase B, recombinase A genes) were used to build a tree for each gene singlewise. Additionally the host genes were concatenated to build an alignment based on the combined markers. Same was meant to be done with the bacterial genes. Concatenation and alignments were performed with Geneious 6.0.5 and tree building was done with the software MEGA 5.0 and confirmed in FastTree 2.1.7. In MEGA 5.0 the bootstrap and Maximum likelihood (ML) methods were conducted. The program was set up to use generalized time-reversible (GTR) models and the ML heuristic method of Nearest-Neighbor-Interchange (NNI). Next step was confirming the MEGA 5 treebuilding by using different software. In FastTree 2.1.7, approximately-maximum-likelihood trees were built using the GTR model of nucleotide evolution. After tree reconstruction, phylogenetic patterns of the host and endosymbionts were confronted in order to assess possible co-speciation. The branches were evaluated with bootstrap support which gives information about the reliability of the certain branch. The nodes were reassembled in 500 replications (MEGA 5). The higher the value in a range between 0 and 100% would be, the more often the node was reassembled in this position and the more probable the position of the defined branch can be assumed.

## 3.11 Sectioning and FISH

In-situ-flourescent-hybridization (FISH) was carried out to visualize the location and interior anatomy of the organs and to detect the presence of general bacteria and *Burkholderia*. Embedding in Technovit® and cross sectioning was performed on the reproductive system of *Lagria rufipennis* including the attached bacteriomes and excluding the ovaries, as displayed in fig.3 C. After dissecting (for description of dissecting process see above) the tissue was fixated in 4% formalin and stored overnight (4°C). Washing out the formalin was done in three repeated steps with

destilled water. Next step was a stepwise dehydration performed in EtOH of a concentration from 70% to 80%, then 90%. Each step was done with 10 min incubation time. The last step was performed in 99% EtOH twice (2 x 10 min) and the sample stored overnight at 4°C. The infiltration with the embedding substance was done in Technovit 8100®. 100 ml basemedium Technovit 8100® was mixed with 0,6 g hardener I to obtain the infiltration solution. For the embedding procedure 15 ml of this infiltration solution were mixed with 0,5 ml of hardener II. Infiltration of the sample was carried out inside a falcon tube during ten minutes on a shaker. As soon as the polymerisation started, the mixture with the sample was poured into an embedding mold by avoiding air bubbles. Underneath the microscope it was placed in the correct direction. To finish a glass coverslide was placed carefully and quickly onto the mold. Overnight storage at 4°C allowed complete hardening. Sectioning was performed with a Rotary Microtome (Thermo Scientific™) in a scale of 6 µm thickness per layer. Slices cut with the glass knife were transfered with a paint brush and placed in waterdrops on a glass slide. These were left at 60°C on a heating plate to dry.

The FISH procedure was done with the probe Eub388 labeled with Cy3 dye (5‘Cy3-GCT GCC TCC CGT AGG AGT-3'), which hybridizes to the eubacterial DNA. Probe Burk_16S marked with the Cy5 label (5’ Cy5-TGC GGT TAG ACT AGC CAC T– 3’) was used to visualize specific *Burkholderia* DNA. 4′,6-Diamidin-2-phenylindol (DAPI) was added to visualize the nuclei of the host tissue for orientation within the tissue structures. The composition of the hybridisation buffer (HB) and washing buffer (WB) is described in table 3. The process started with determining the appropriate samples on the selected glass slide under the microscope. 170µl of HP and 10µl per probe (10pmol/µl) were added to the slide to cover the samples. The slide was incubated at 50°C for 90 min conserving humidity. Meanwhile the WB was heated up to 50°C for the following washing steps. First the HB was removed from the samples, then the slide was covered with 200µl of WB. After removing the WB, this step was repeated followed by an incubation step of 20 min at 50°C. Distilled water was used to clean the surface twice after removal of

the WB. The samples were left to dry in darkness. VectaShield® (Linaris) was used for mounting and a cover glass to protect the samples.

A fluorescence microscope (Zeiss) with an AxioCam HRc and the software AxioVision Release 4.8.1 were used to visualize the FISH results. The set ups were chosen so Cy3 should appear yellow green (Excitation 550 nm, Emission 570 nm) and Cy5 as red signal (Exitation 650nm, Emission 670 nm). DAPI is labeling the nucleic acid in the tissue so the eukayotic host cells will be visualised by their blue stained nuclei since DAPI has it's emission maximum at 461 nm.

Table 3: Components and Volumes of HP and WB for FISH

| | 1 ml HB – Volume in µl | 1 ml WB – Volume in µl |
|---|---|---|
| **Aqua dest.** | 800 | 950 |
| **5M NaCl** | 180 | 20,4 |
| **1M TRIS/HCl, pH 8** | 20 | 20 |
| **0,5M EDTA** | - | 10 |
| **10% SDS** | 1 | 1 |

# 4.RESULTS AND DISCUSSION

## 4.1 HOST MORPHOLOGY AND BACTERIAL CONTENT

Figures 4 to 8 illustrate the dissection process of insect specimens to recover the structures of interest. The dry condition of *Lagria rufipennis* (fig 4) can be observed by comparing the dark body fat to the bright yellow and soft conditioned body fat of *Lagria nigricollis* (fig. 5), which is in better condition. In cases of male samples there were as expected no bacteriomes detected. As fig. 8 shows, in some cases there were tube-like structures extracted. *Lagria* sp. (South Africa) is not shown. *L. hirta* and *L. villosa* were not dissected.

Fig. 2 shows Stammer's [7] illustration of the whole reproductive system with bacteriomes included. In this case he described the organs and anatomy of *Lagria hirta*. Two types of bacteriome structures were found. "Ovipositor sacs" enclosed in the ovipositor and "intersegmental sacs" as pouches connected on each side of the ovipositor. In both tissues he could observe bacteria with the same morphology, but not determine genus or species by that time. As displayed *Lagia nigricollis* shows the

same bacteriome structure described as “Intersegmentaltaschen”. Morphologically the findings accord the anatomy of *Lagria hirta*. This was observed in *Lagria rufipennis* as well which conforms findings by Stammer in this species. The pouches of *L. rufipennis* were far more flat and transparent then those of *L. nigricollis,* what could have originated from the dry condition (or other factors like e.g the age by time of death) of *L. rufipennis*. The PCR products of *L. nigricollis* were richer in content of the bacterial DNA due to the better sample quality. In both – *L. rufipennis* and *L. nigricollis*- bacterial DNA content could be detected after PCR on the reproductive system samples what indicates the presence of an enclosed bacteriome (ovipositor sacs). In both species the identity was determined as *Burkholderia gladioli.* It also could be the case that not an organ, but, traces of bacteria containing mucus were trapped in the reproductive duct after laying eggs and egg smearing with the bacteria containing mucus.

Fluorescent-in-situ-hybridization (FISH) was conducted on *L. nigricollis* to visualize the bacterial content inside the organs. For this purpose, cross sections of the reproductive system are shown. In fig. 3 it is visible that pouches are filled with bacteria marked by the probe specific for general Bacteria. In the conical part of the ovipositor a green fluorescent signal shows bacteria too. Blue stained nuclei (DAPI labeling) of the host tissue give a reference for orientation in the structures. These observations meet the expectation since they match results of *Lagria hirta* [6].

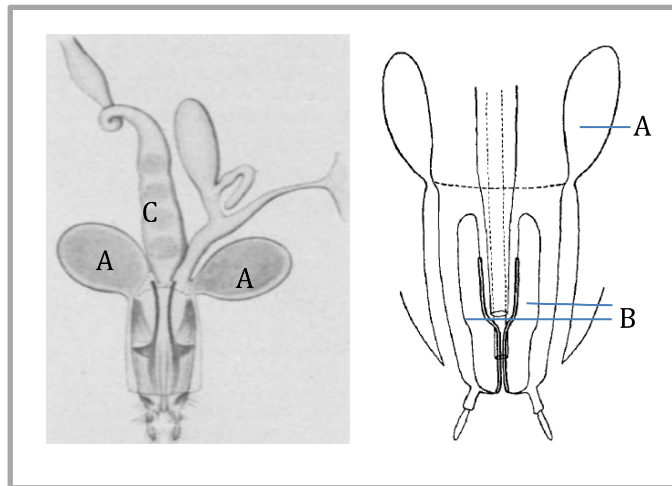


Fig. 2: Intersegmental sacs A ovipositor sacs B, gut lumen C, of *Lagria hirta* [7]

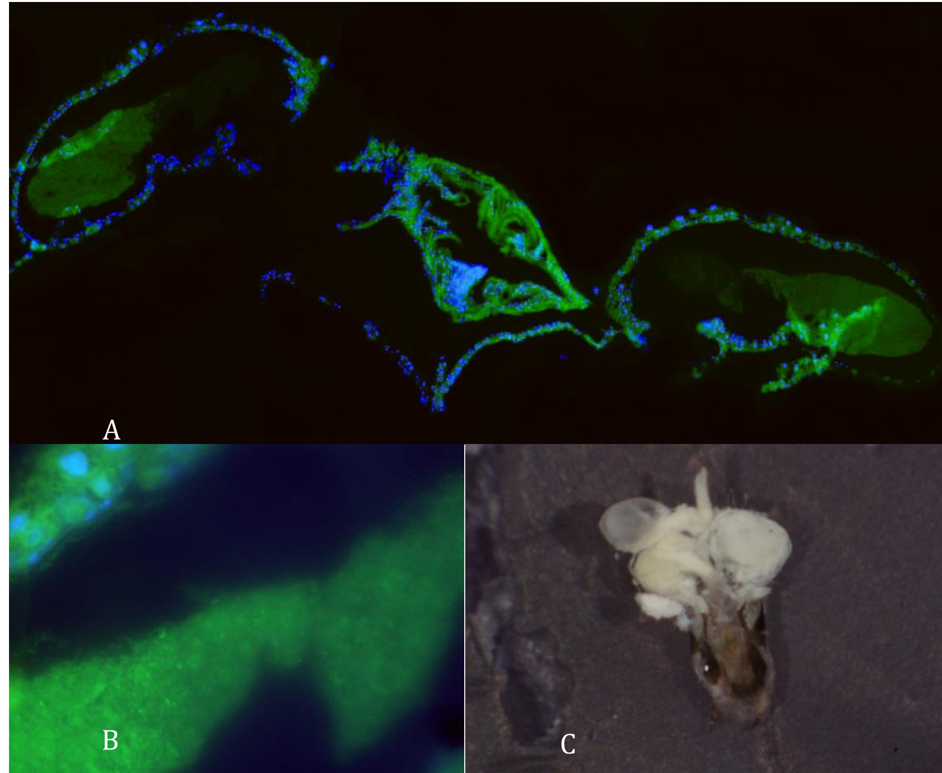


Fig. 3:**A** 5xMag of a cross section of *Lagria rufipennis'* reproductive system labeled by FISH (probe Eub338-Cy3 gives green signal and DAPI gives blue signal); **B** 63xMag/1,4 Oil, blue stained nuclei, green spotted area of bacterial containing secretion within the bacteriomes ; **C** dissected reproductive system of *Lagria rufipennis*

The results of the FISH method are shown in fig. 3 A and B. Fig. 3 C shows the dissected reproductive system of *Lagria rufipennis.* The microscopic 5x magnification of a cross section labeled by FISH (probe Eub338-Cy3 gives green signal and DAPI gives blue signal) shows two pouches lateral to the ovipositor. The red labeling by the probe Burk_16S-Cy5 is missing which could either mean that no *Burkholderia* is present or the labeling process did not proceed as expected. Since *Burkholderia* was confirmed in all of the *Lagria rufipennis* samples further tested by diagnostic PCR, it is highly probable that the results are due to failed hybridization or that the dye could not be visualized. Fig. 3 B is a close up (63xMag) of the structure, blue stained nucleic acid regions represent a part of the host epithelium, while the green spotted area corresponds to bacterial cells within the pouches. The smooth

green areas in A and B are not bacteria containing structures, more likely they appear as autofluorescence within the tissue. Interior structures of the ovipositor could contain the bacteria as well as in *L.hirta*, but this remains uncertain. Because of the autofluorescence it is difficult to distinguish the bacterial DNA.

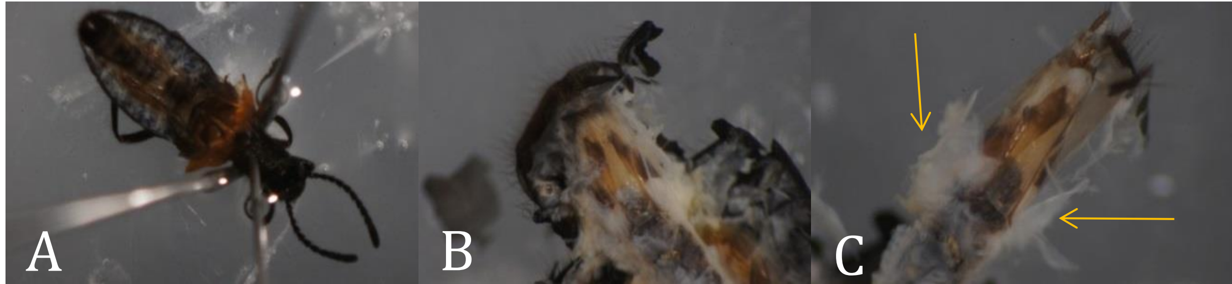

Fig. 4: **A** 1.1 *Lagria rufipennis,* **B** reproductive system, **C** bacteriome tissue (arrows)

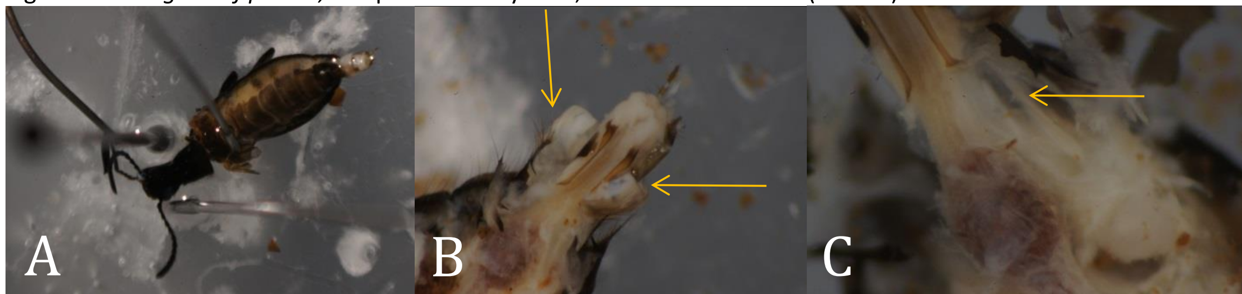

Fig. 5: **A** 2.1 *Lagria nigricollis,* **B** reproductive system with bacteriome tissue (arrows), **C** tube (arrow)

Fig. 6: **A** *Macrolagria rufobrunnea,* **B, C** reproductive system

For *Macrolagria rufobrunnea* (fig. 6) no bacteriome structures could be observed. Either the tissue was folded in such way they could not be distinguished to other fine and transparent tube-like structures or there was nothing as bacteriome organs existing in this case. The whole reproductive system was extracted and further tested to definitely determine the presence of bacteria in the tissue. The reproductive system sample of *Macrolagria rufobrunnea* showed irritating results after a eubacterial 16S rDNA and gyr B PCR (data not shown). Eubacterial 16S rDNA was extremely slight in appearance of the band and could not be amplified again, this therefore leads to the assumption that a contamination during PCR preparation could be the

cause. The primers for the gyrase B PCR reaction amplified a *Burkholderia* gyrase B gene fragment which could not be identified after sequencing, because of no informative sequencing results. After several tests of different dilutions it was clear, that no bacterial content could be detected based on the eubacterial 16S rDNA and recombinase A gene amplification, which meets the fact of the absence of bacteriome structure. The results of the gyrase B gene must be then explained by mixing up the samples or a contamination with *Burkholderia.* A confusion while pipetting is more probable than a contamination, otherwise the primerpair for the general bacterial 16S rDNA would have managed to amplify a fragment consistently in a usable amount far earlier in the experiment as long as there is no overload and consequently inhibition by (host) DNA. Also the dissection was performed with no notable gut ripping and following contamination with bacteria of feces or from the gut lumen and the negative controls showed results allowing trust in the PCR reaction. A test PCR was conducted with host specific 28S rDNA primers to see whether the sample contains DNA at all. Fragments could be amplified and no inhibition was the case in the undiluted sample. If there is bacterial DNA it could be the case that it is incorporated in an extreme low amount, which cannot be verified without other samples of this species. Also the sample or genus is not included in Stammer's collection [7], hence the present findings cannot be compared. Considering this it can not be confirmed whether *Macrolagria rufobrunnea* carries bacteria in the reproductive structures.

Fig. 7: **A** *Arthromacra viridissima,* **B, C** reproductive system

For *Arthromacra viridissima* (fig. 7) no bacteriome structures could be identified. There were assumptions on regions of the tissue from the reproductive system, therefore a sample from the reproductive system was taken to investigate if its identity could be a high amount bacteria containing structure. This species was not described by Stammer, but, another species of this genus is listed in his descriptions named *Arthromacra aena* [7]. For this sample he could not identify any bacteriome structures either. Because of his findings, it can be assumed that

*Arthromacra viridissima* possibly does not develop structures to harbor endosymbionts. At least missing bacteriomes can be now confirmed for two representatives of the genus *Arthromacra* namely *A. aena* and *A. viridissima*. Stammer could not say whether there is bacterial symbiosis in this species. The insights into molecular data do not show the presence of *Burkholderia* endosymbionts neither in the assumed bacteriome structure nor in the reproductive system. The gyrase B and recombinase A gene amplified with *Burkholderia* specific primers led to no bands after gelelectrophoresis. Nonetheless there were eubacterial 16S rDNA fragments detected after one single PCR with the primers fd1 and rp2. The bacterial identity could not be determined after sequencing because of low sequence quality. It might have occurred as a contamination issue. Probable is a contamination while dissecting with bacteria from feces or the gut lumen or any other body tissue. Although ripped gut was not notable while dissection it is possible scissures in the epithelia were caused. The body sample was tested to detect eubacterial 16S rDNA at the beginning of the experiment and showed the existence of bacteria. Either these bacteria were detected in the bacteriome and reproductive samples or the contamination was caused by other bacteria in the environment while PCR preparation. It still remains unclear and interesting whether *Arthromacra viridissima* has evolved a different way of harboring endosymbionts in the reproductive system without developing visible bacteriomes (pouches) and without the uptake of *Burkholderia* species. To clearify this, a more intense investigation on the bacterial content is necessary. A higher dilution would maybe contribute in enhancing the DNA content in the PCR products, in order to exclude PCR inhibition due to a nucleic acid overload. Also a higher sample size would allow grounded statements to this issue.

Fig. 8: **A** *Ecnolagria sp. (grandis?),* **B** reproductive system, **C** bacteriome tissue

*Ecnolagria grandis* is described by Stammer as a bacteriome carrying species. The specimen of this experiment was only identified to the genus level (fig. 8). It was uncertain whether it can be *E. grandis* by classifying morphological attributes before dissection. Stammer found structures of long, tip-branched. The interior anatomy of the present specimen *Ecnolagria sp.* did show these anticipated structures marked with arrows in fig. 8 *Ecnolagria sp.* showed positive results for endosymbionts in the tested tissues. By cloning and sequencing the identity was determined as *Burkholderia gladioli.*

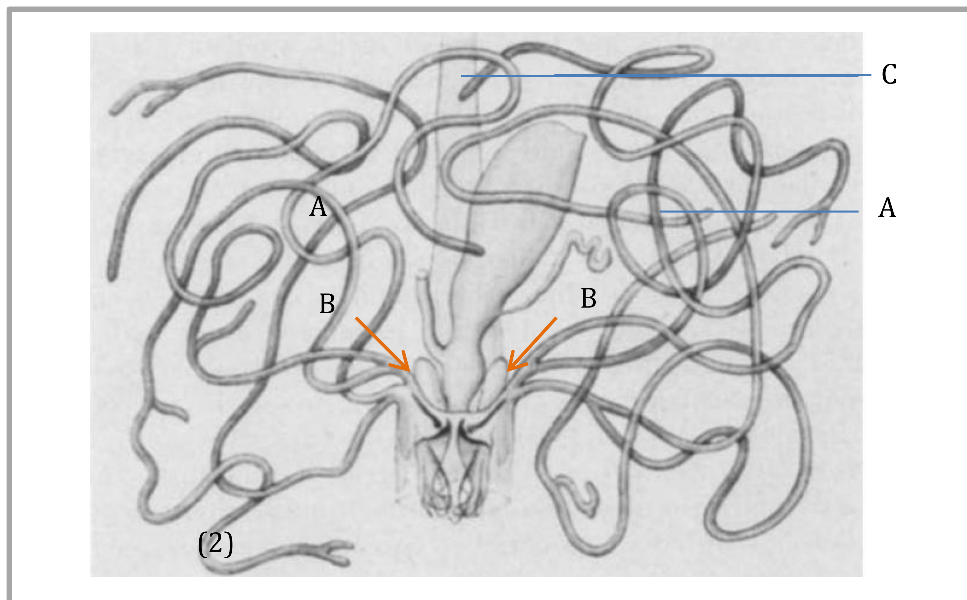


Fig. 9: A intersegmental sacs, B ovipositor sacs, C gutlumen, of *Ecnolagria grandis* [7]

*Casnoidea* nov. spec. is an examined sample of Stammer. It could be seen as a reference for other species in the genus *Casnoidea.* The sample *Casnoidea dimidiata* dissected in this experiment (data not shown) did not have any bacteria accommodating structures. Since this was a male specimen we can not conclude about the presence of bacteriomes.

The sample number of *Macrolagria rufobrunnea, Ecnolagria dimidiata, Arthromacra viridissima* was too low for reliable observations of the organs and the containing symbiont's DNA as there was mainly only one sample available. Only two species were examined in a higher amount of four samples per species (*Lagria rufipennis, Lagria nigricollis*) and delivered reliable data. Living samples would contribute to research on other possible questions of the transmission mode, same as on biochemical, molecular and ecological issues connected to the establishment of the symbiosis. In table 4 the summarized data of the morphological and molecular analysis on the bacterial content are displayed.

Table 4: Summary of morphological observations on the Lagriinae hosts and molecular analysis of the bacterial content

| ID | Species | Bacteriome | Reproductive system | Eubacterial 16S rDNA | Gyr B | Rec A | Bacterial identity |
|---|---|---|---|---|---|---|---|
| **1** | *Lagria rufpennis* | isolated | isolated | X | X | X | *Burkholderia gladioli* |
| **2** | *Lagria nigricollis* | isolated | isolated | X | X | X | *Burkholderia gladioli* |
| **3** | *Macrolagria rufobrunnea* | not existing | isolated | - | X* | - | no bacteria |
| **4** | *Arthromacra viridissima* | not existing | isolated | X | - | - | not identified |
| **5** | *Ecnolagria sp. (grandis?)* | isolated | isolated | X | X | X | *Burkholderia gladioli* |
| **6** | *Casnoidea dimidiata* | not existing | isolated | - | - | - | no bacteria |
| **7** | *Lagria sp.* (South Africa) | not existing | isolated | - | - | - | no bacteria |
| **8** | *Lagria hirta* (provided data) | not dissected, but confirmed | not dissected, but confirmed | X | not tested | not tested | *Burkholderia gladioli* |
| **9** | *Lagria villosa* (provided data) | not dissected, but confirmed | not dissected, but confirmed | X | not tested | not tested | *Burkholderia gladioli* |

X* Data is not reliable due to the explanations of morphological observations on *Macrolagria rufobrunnea* and its molecular analysis of the bacterial content. The data could not be verified and occurred only once- assumed as contamination (for more details see description of *Macrolagria rufobrunnea* above).

## 4.2 Phylogenetic analysis

### 4.2.1 Phylogeny of the bacterial symbionts

The reconstruction of the phylogenetic tree based on the eubacterial 16S rRNA gene of various *Burkholderia* ingroup species and the tested endosymbiotic species show the results displayed in fig.10. *Ralstonia insidiosa* was chosen as the outgroup. The *Burkholderia* species integrated from the GenBank database originate from different environments, such as plants (e.g. pathogens of *Oryza sativa, Allium* and *Gladiolus – B. cepacia, B. plantarii, B. vietnamiensis; B. gladioli, B. glumae),* soil (soil-borne rhizospherical bacteria – *B. ambifaria, B. oklahomensis, B. tropica)* and human pathogens (Cystic fibrosis - *B. stabilis).* Estrada-de los Santos et. al [10] evaluated the named species as belonging to the saprophytic and plant associated group A except *Burkholderia tropica*, which is is found in group B. The clade of *Burkholderia gladioli* strains forms unsurprisingly a monophyly with a high evaluation (bootstrap values 0,800-0,899), while the other species form clades which have mixed content of plant associated, soil-borne or human pathogens. However the bootstrap method results indicate a strong validity of their position (bootstrap values 0,900-1,000). Within the monophyletic group of *Burkholderia gladioli* strains the evaluation of the positions marked by the colored asterisks show lower certainty. In fact 25% of the values range lower than 0,5. Half of the positions don't show higher values than 0,799. The symbiotic strains intermingle with soil-borne and plant pathogenic strains. This leads to the result of no monophyly formation within the symbiotic bacteria group, but a paraphyletic clade including all the investigated symbiotic *Burkholderia* and leaves a high probability of unclearified node positioning.

Originally the other bacterial genes recA and gyrB were meant to be included into the phylogenetic tree by concatenation of all three. The amount of tested species did not deliver as many useful high quality sequences as the eubacterial 16S rRNA gene. Although the 16S rRNA is one of the most common genes to be used as a tool for distinguishing organisms and their phylogenetic history, it can be difficult to resolve the phylogenetic relationships among the *Burkholderia* strains based only on this marker. The loss of informative variable regions outside the

available sequence length (949bp) can contribute to low resolution and/or false positioning of samples within the clades.

### 4.2.2 Phylogeny of the insect host

Fig. 11 shows the phylogenetic reconstruction via 18 and 28S rRNA gene concatenation of several tested species and included ingroup species from the Lagriinae subfamily. COI was left out due to short length or low quality of the sequences. The two genes used in the tree are good markers for deep phylogenetic relationships, but are not as useful for information of closely related individuals. COI would have clearified more accurate for example the genetic distance of *L. rufipennis* and *L. nigricollis*. In the single COI gene tree they form a sisterclade (data not shown), which is not the case in the presented phylogeny. The tree shows that the species carrying symbiotic *Burkholderia*, i.e. *L. rufipennis. L. nigricollis, L. hirta* and *Ecnolagria sp.,* are grouped separately from those that did not harbour these bacteria, i.e. *Macrolagria rufobrunnea* and *Arthromacra viridissima (Casnoidea dimidiata* and *Lagria sp.* cannot be arranged in this comment, hence they were male specimen). The high bootstrap value 97,2% of the tested hosts with *Burkholderia* symbionts suggests that it is highly probable to see those species as close relatives compared to the non bacteria harbouring species. It would be interesting to include the data of *Lagria villosa,* which was not available at the point of experimentation, but whose data is currently in progress. It can be expected to find this species grouped within the symbiotic Lagriids. But, it must be noted that up to this point the available information is not enough to conclude on the monophyly of the symbiont-containing clade. There is a lack of information about the bacterial and bacteriome content in *Cerogria bryanti* and female *Lagria sp.*. Confirming the presence of symbiotic Burkholderia in these species would support the monophyly of Burkholderia-associated Lagriids. However the current results do not falsify this hypothesis, since the Lagriid hosts with positive results group together in one clade and do not intermingle with species where *Burkholderia* was not found.

### 4.2.3 Co-Phylogeny

In fig. 12 both trees described before were co-aligned to visualize the relationships of symbiont carrying insects and their particular endosymbiotic bacteria. There is no clear co-speciation visible. In the 16S rRNA tree the symbionts form a sisterclade for *Lagria rufipennis* and *Lagria nigricollis,* while the host tree shows no such grouping. As mentioned above this could be an incident of misplacement based on the missing data of the COI genes. Symbionts of *Ecnolagria sp*. And *Lagria villosa* appear as units of the same clade, while in the host phylogeny this relationship cannot be estimated, because *L.villosa*'s 18S and 28S data was not available for this version of tree building. *Ecnolagria sp.* seems to be closely related to the South African species *Lagria sp.*, which as mentioned before is a sample not clearified in terms of the female reproductive system and its bacterial content. The *Lagria hirta* endosymbiont appears in a earlier branched position than all other symbiotic bacteria, which is not reflected in the host's reconstruction. There *L. hirta* is embedded closer to the other *Burkholderia* associated Lagriids. The issue of probable false positions and inaccurate resolution in the host phylogeny is again occurring here. However the symbiotic host species group definitely closely related, while the endobacteria are highly spread in their phylogeny and intermingling with other *Burkholderia* species which were not detected as endosymbionts of arthropods so far. The availability of Lagriid sequences in the database was limited and results in a lower sampling amount for the host tree than for the bacterial tree, what can open out into this phenomenon. Hence the findings of strict vertical transmission cannot be confirmed by these results. For this it would have been nescessary to see a co-cladogenesis of symbionts and hosts. In fact the results lead to the assumption of seeing an occasional horizontal transfer of the symbiotic content to the Lagriid's offspring generation. These findings seem to meet the current observations in other experiments on Lagriids and their endosymbionts [6].

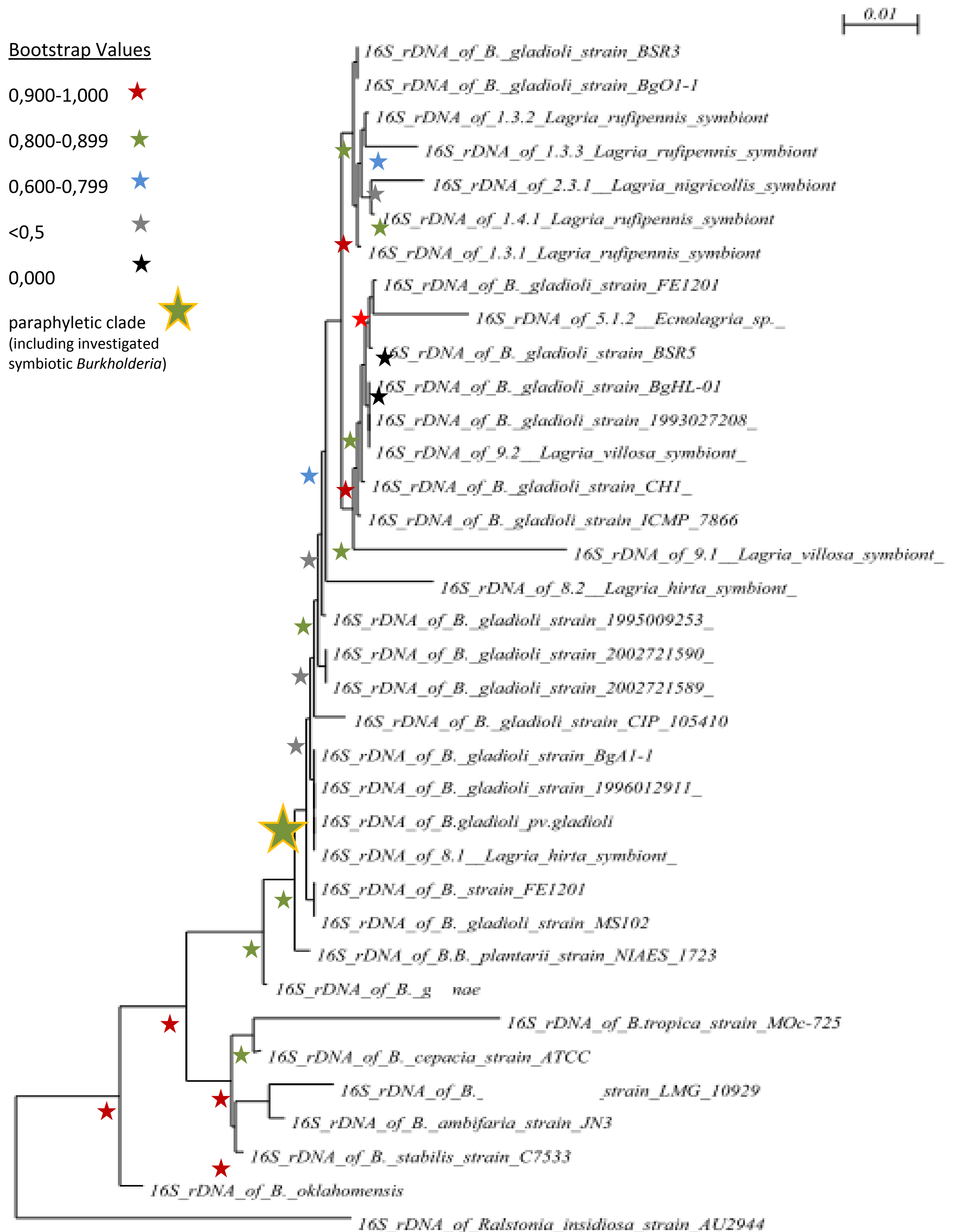


Fig 10: Phylogenetic tree based on eubacterial 16S rDNA (alignment with 949 bp of total lenght); created with the software FastTree 2.1.7 (for setting details see methods section); bootstrap values are represented by asterisks; bar shows number of expected substitutions per site under GTR model (genetic distance)

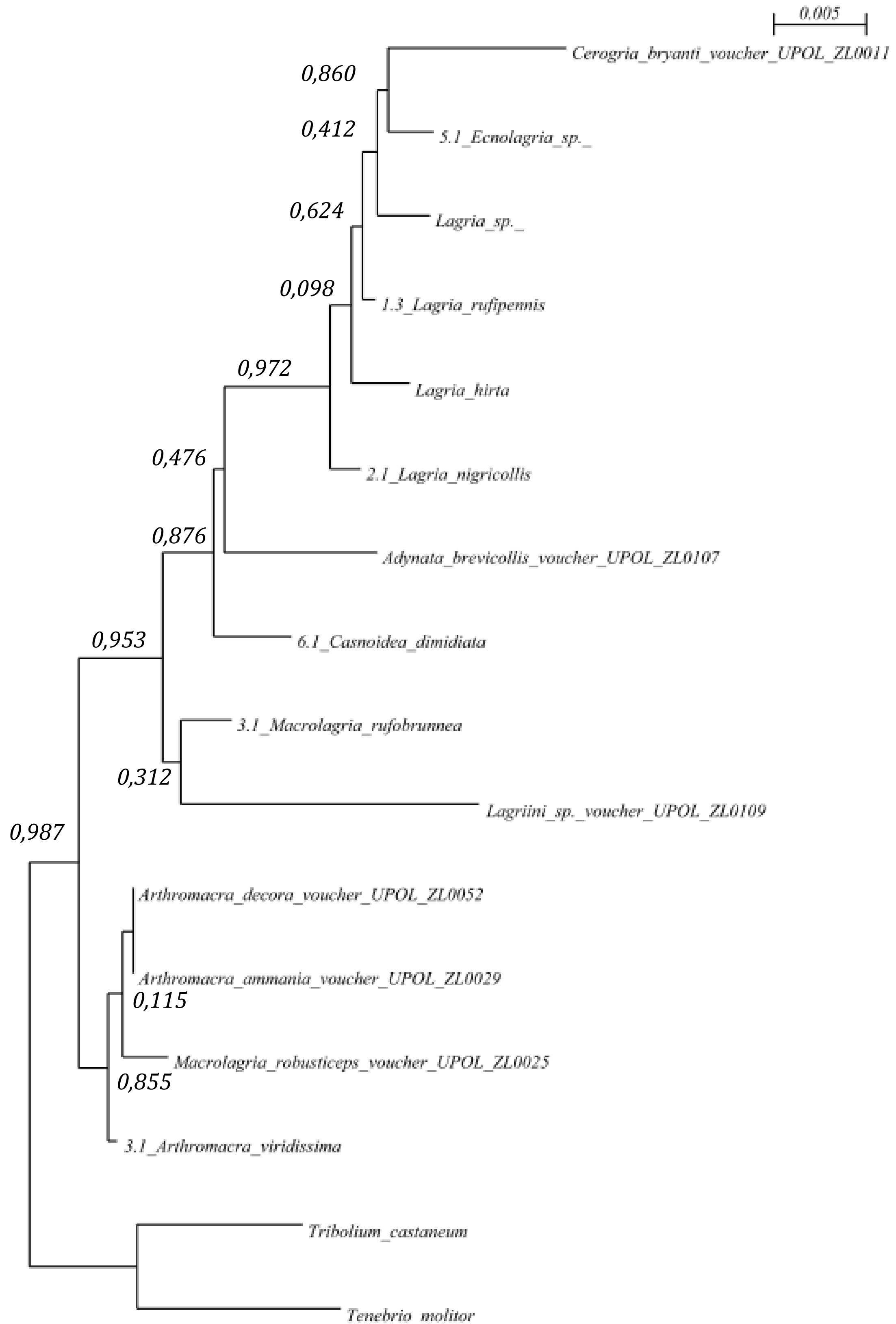


Fig 11: Phylogenetic tree based on concatenated 18S rDNA (alignment with 1261 bp of total length) and 28S rDNA (alignment with 396 bp of total lenght); created with the software FastTree 2.1.7 (for setting details see methods section); bootstrap values are displayed on each node; scale bar shows number of expected substitutions per site under GTR model (genetic distance)

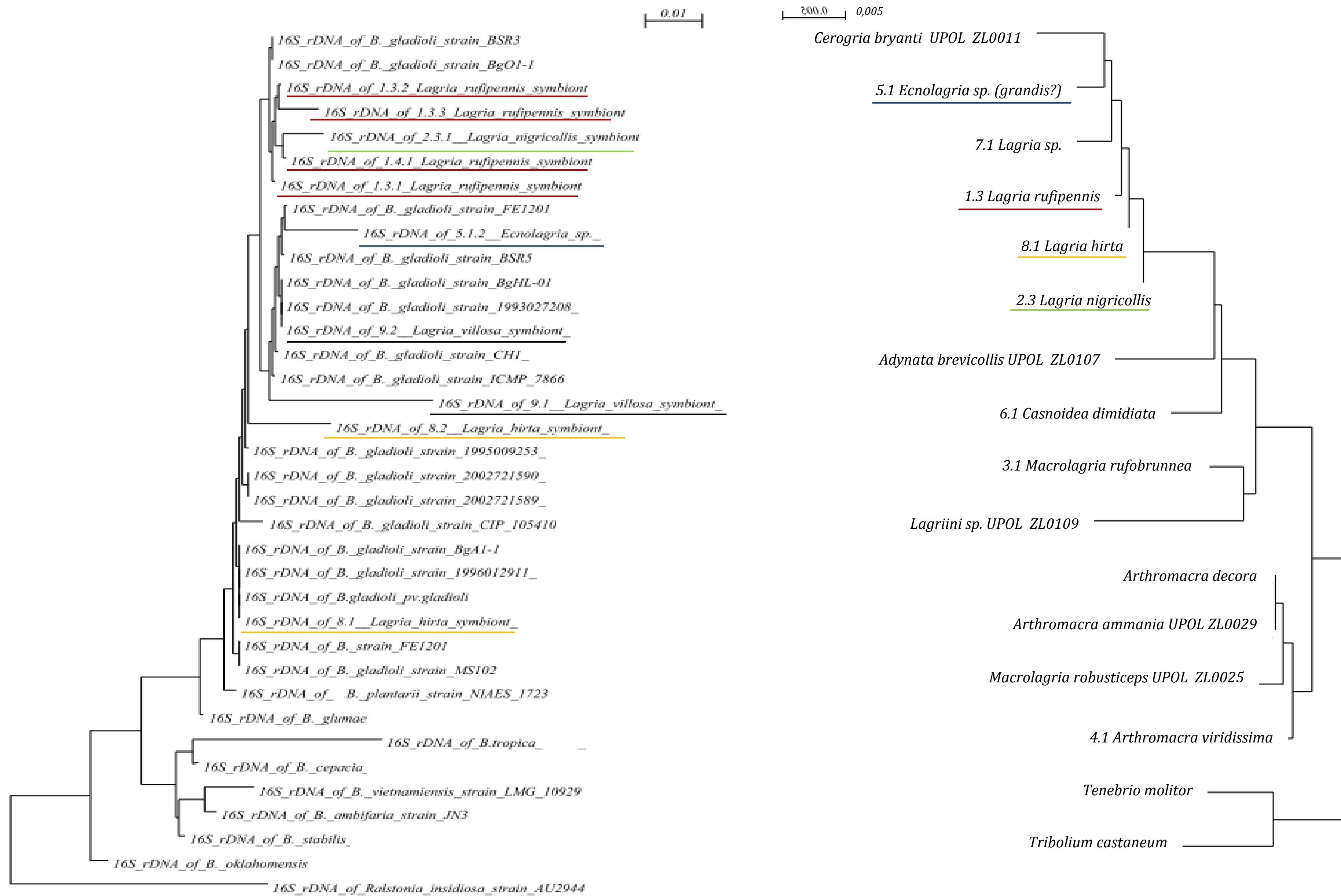


Fig. 12: Co-phylogeny of the bacterial and host phylogenetic trees; 16S Eubacterial rDNA (left) and concatenated host 18S and 28S rDNA (right); underlined samples represent symbiont acommodating species and their symbionts; coloration displays affiliation; bar shows number of expected substitutions per siteunder GTR model (genetic distance)

## 4.3 Discussion and Outlook

The identity of the endosymbionts seems to be strictly limited to the strains of *Burkholderia gladioli*, which is known as a plant pathogen. A mode of occasional horizontal transmission must be involved as reflected from the phylogenetic reconstruction of the bacteria. The specific mechanism, however, is not understood yet. The maintance and establishing mode of symbiosis in every generation needs to be elucidated more intensively, because it might be possible to find a combined mode of vertical and horizontal transmission in this example. A closed and intimate relationship between host and symbiont maintained by strict vertical transmission would have supported a co-speciation, which is not the case. Environmental factors such as plant, root and soil associated *Burkholderia* species can contribute to the maintenance of in this case experimentally proven high specificity of the symbiont's identity. Since the Lagriids depend on plants to feed on, it should be clarified whether these plants mediate the association. Due to the wide distribution of the host species, it was unexpected to find only *Burkholderia gladioli* in all the tested symbiotic insects. This could be reflective of environmental acquisition with a highly specific mechanism for symbiont recognition and maintainance. It is crucial to allow more deep insight into the bacterial DNA content to achieve a high quality statement of their identity and real diversity. More genes and informative sequences must be consulted in the experimental procedure.

The insights into the Lagriinae-Burkholderia symbiosis can contribute to studies on extracellular endosymbionts of insects. In *Lagria hirta* it is experienced so far that aposymbiotic insects show growth and development retardation, but still can be examined. This fact is a fortune for the range of possibilities regarding the upcoming experimtal design and should be confirmed for more Lagriid species. Culturable bacteria allow creating genetically manipulated symbionts in order to reinfection of the host and pinpoint the involved biochemical paths of symbiosis establishment. The tools of gene silencing and knockout can vary from RNAi to homologue recombination in order to disrupt functional genes of interest. Especially a possible mode of partly horizontal transmission should get attention, as those mechanisms are unknown. Since there could be no monophyletic origin

of the symbiotic bacteria determined by tree reconstruction (fig 10), the investigation of probable but so far undetected molecular symbiosis factors can lead to better understanding of the Lagriid association. The molecular mechanisms detected in Alydidae-*Burkholderia* symbiosis serves as an example for orientation, as it is a thoroughly described case of a *Burkholderia*-insect association.

Kikuchi et. al is conducting research on Alydidae-*Burkholderia* symbiosis where the symbiont is harboured extracellularly in midgut crypts. Function of the symbiont was nailed down as facilitating growth and occasionally conferring organophosphorus insecticide resistance [19]. Recently it could be observed that bacterial cytoplasmic granules of polyhydroxyalkanoate (PHA) accumulate in the bacteria what leads to protection from environmental stresses. The results of the stinkbug study indicate, that *Burkholderia* symbiont's PHA synthesis genes are crucial for symbiotic association and confer stress resistance to the host [19]. In further studies on bacterial symbiotic factors, cell wall components of the *Burkholderia* symbiont were found to mediate the establishment of the symbiosis [20]. The findings of a highly specific association to *Burkholderia* led to the hypothesis of an insect analogue to some highly specific plant-microbe associations [21].

The idea of an analogue was suggested due to his solved phylogenetic enigma of stinkbug-*Burkholderia* symbiosis as the different species and strains of the *Burkholderia* genus do not form a phylogenetic relationship of cospeciation or cocladogenesis with the Alydidae host lineage. A similar pattern of intermingled non-symbiont and symbiont *Burkholderia* species occurred as an enigma also in the present study. In the legume-microbe same as in the stinkbug-microbe relationship a sophisticated way of molecular communication to establish the symbiosis has evolved [22]. It would be interesting to follow the way of establishing a Lagriid-microbe association as well. The interesting Alydidae-*Burkholderia* association research can hold as a template for further experimental design to look for similar or deviating biochemical (e.g. cell wall composition) and ecological correlations (e.g. function of the symbiont for the host) in the Lagriid-microbe interaction.  It would thereby be possible to assess it there are

common or specific factors mediating the two endomicrobial and extracellular symbioses (Lagriid-*Burkholderia* versus stinkbug-*Burkholderia).* Despite of the two different transmission routes (vertically transmitted bacterial immigration versus horizontal transfer by nymphal uptake). Kikuchi's solved phylogenetic enigma also provides a base for the discussion of surprising molecular data of the Lagriinae host associated to the strictness of the observed vertical transmission mode.

A further interesting question about this system relates to the stage of devolpement of the holometabolic Lagriid in which the division into symbiont carring females and uninfected males occurs. The crucial step of bacterial migration through the egg shell and the formation of invaginations before hatching is so far assumed to be present in all larvae [6]. A possible next step would be to get access to the molecular and biochemical configurations which could drive a different process in males and females and are potentially relevant factors in the symbiosis. This could be conducted by transcriptomic research on the larvae in different stages, as well as in pupae, to elucidate the changes in the expression levels of genes which are connected to this division and the control of the bacterial content in females and analysis of the chemical conditions and composition of the egg smear to see what interdependencies could lead to fix the symbiosis and developing bacteriomes in the female after egg smearing.

The next question is if resistance to insecticides and other environmental stress factors or developmental benefits play roles for the insect host. Some representatives of the genus *Burkholderia* are capable of conferring different benefits to plants as nitrogen-fixing, nodulation, growth promotion, immunity to diseases [10]. An investigation of molecular interactions between the symbiosis partners with the particular environment would be necessary. On the molecular level it is expected to find mechanisms, which allow biochemical communcation to stabilize and establish the specifity. These factors may also aid in selecting among other bacterial species possibly taken up from the environment. To find a molecular repressing or specifity-mediating mechanism in the *Burkholderia gladioli* as it was discovered before in other symbiosis examples (e.g. legume-microbe association) against all other possible endosymbionts around, would

elucidate how in despite of such a broad geographical distance between the host species this high specifity to *Burkholderia gladioli* can be maintained over the host's evolutionary history in the Lagriinae family. Experimentally the adult female could be infected with different *Burkholderia* to see, whether *B. gladioli* is allowing the presence of a consortium or outcompeting all other identities. Following molecular analysis of the egg shell surface should show how diverse the bacterial content can be compared to the wild-type composition and how this has influence on the vital functions of the larvae. The development of the bacteriomes in the maturing organism could be monitored in terms of how the bacterial content is composed after the pre-treatment with a mixture of different *Burkholderia* species. In case of finding different bacterial species a comparison of expression patterns in treated and wild-type hosts could give then insight into unknown probable factors mediating the establishment of the specific Lagriid - *Burkholderia gladioli* asssociation.

Conclusions

All observed results of this experimental study can be summed up as follows: i) Four out of six female species of Lagriinae had bacteriome structures as found in *Lagria hirta,* while two species could not be estimated, because the samples were males and showed no such structure. Those species are *L. rufipennis, L. nigricollis, L.villosa*, and *Ecnolagria sp.,* which are geographically distant in origin. ii) The endobacterial content in these organs seems to have the same identity as in *Lagria hirta,* which is *Burkholderia gladioli,* but a certain level of diversity is still occuring due to the presence of different strains (see table 5 in appendix). Problems of fragment lenght and quality contributed to a loss of infomative sequences (see section of troubleshooting). iii) Symbiont positioning in the phylogenetic reconstruction based on the 16S rRNA gene does not support a strict vertical transmission of endosymbiotic *Burkholderia* species. They intermingle with soil-borne and plant-pathogenic species. iv) Symbiont carrying host Lagriids seem to be closely related and group in one clade in the phylogenetic tree based on 18S and 28S rRNA data. The positioning of the branches does not match the positions of the particular hosts – a co-speciation is not visible. Since it is not known whether *Cerogria bryanti* accomodates bacteria in the same manner as the other clade members or secondarily lost this feature, no statement about the association to *Burkholderia* as an apomorphy of the clade can be made to date.

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Datum und Unterschrift

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# IV. APPENDIX

Table 5: First five BLAST results for all the tested bacterial content oft he particular host

BLAST result for 16S rDNA of 1.3.1 *Lagria rufipennis* symbiot

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli BSR3 strain BSR3* | 43% | 1e-124 | 99% | NR_102847.1 |
| *Burkholderia gladioli strain BgO1-1* | 43% | 1e-124 | 99% | JX566502.1 |
| *Burkholderia gladioli strain BSR3* | 43% | 1e-124 | 99% | JF431409.1 |
| *Burkholderia gladioli BSR3* | 43% | 1e-124 | 99% | CP002600.1 |
| *Burkholderia gladioli BSR3* | 43% | 1e-124 | 99% | CP002599.1 |

BLAST result for 16S rDNA of 1.3.2 *Lagria rufipennis* symbiot

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli BSR3 strain BSR3* | 27% | 2e-126 | 99% | NR_102847.1 |
| *Burkholderia gladioli strain BgO1-1* | 27% | 2e-126 | 99% | JX566502.1 |
| *Burkholderia gladioli strain BSR3* | 27% | 2e-126 | 99% | JF431409.1 |
| *Burkholderia gladioli BSR3* | 27% | 2e-126 | 99% | CP002600.1 |
| *Burkholderia gladioli BSR3* | 27% | 2e-126 | 99% | CP002599.1 |

BLAST result for 16S rDNA of 1.3.3 *Lagria rufipennis* symbiot

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli BSR3 strain BSR3* | 44% | 1e-124 | 99% | NR_102847.1 |
| *Burkholderia gladioli strain BgO1-1* | 44% | 1e-124 | 99% | JX566502.1 |
| *Burkholderia gladioli strain BSR3* | 44% | 1e-124 | 99% | JF431409.1 |
| *Burkholderia gladioli BSR3* | 44% | 1e-124 | 99% | CP002600.1 |
| *Burkholderia gladioli BSR3* | 44% | 1e-124 | 99% | CP002599.1 |

BLAST result for 16S rDNA of 1.4.1 *Lagria rufipennis* symbiot

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli BSR3 strain BSR3* | 79% | 0.0 | 99% | NR_102847.1 |
| *Burkholderia gladioli strain BgO1-1* | 79% | 0.0 | 99% | JX566502.1 |
| *Burkholderia gladioli strain BSR3* | 79% | 0.0 | 99% | JF431409.1 |
| *Burkholderia gladioli BSR3* | 79% | 0.0 | 99% | CP002600.1 |
| *Burkholderia gladioli BSR3* | 79% | 0.0 | 99% | CP002599.1 |

BLAST result for 16S rDNA of 2.3.1 *Lagria nigricollis* symbiot

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli BSR3 strain BSR3* | 24% | 6e-107 | 98% | NR_102847.1 |
| *Burkholderia gladioli strain BgO1-1* | 24% | 6e-107 | 98% | JX566502.1 |
| *Burkholderia gladioli strain BSR3* | 24% | 6e-107 | 98% | JF431409.1 |
| *Burkholderia gladioli BSR3* | 24% | 6e-107 | 98% | CP002600.1 |
| *Burkholderia gladioli BSR3* | 24% | 6e-107 | 98% | CP002599.1 |

BLAST result for 16S rDNA of 5.1.2 *Ecnolagria sp.* symbiot

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli strain XSJ6* | 57% | *2e-152* | 99% | *KF527218.1* |
| *Burkholderia gladioli BSR3 strain BSR3* | 57% | *2e-152* | 99% | *NR_102847.1* |
| *Burkholderia gladioli strain BgYP-5* | 57% | *2e-152* | 99% | *JX566505.1* |
| *Burkholderia gladioli strain BgA1-1* | 57% | *2e-152* | 99% | *JX566504.1* |
| *Burkholderia gladioli strain BgHL-01* | 57% | *2e-152* | 99% | *JX566503.1* |

BLAST result for 16S rDNA of 8.1 *Lagria hirta* symbiot (cultured)

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli strain BgA1-1* | 100% | *0.0* | 99% | JX566504.1 |
| *Burkholderia gladioli BSR3 strain BSR3* | 100% | *0.0* | 99% | KC841439.1 |
| *Burkholderia gladioli strain BgYP-5* | 100% | *0.0* | 99% | KC702728.1 |
| *Burkholderia gladioli strain BgA1-1* | 100% | *0.0* | 99% | AB680484.1 |
| *Burkholderia gladioli strain BgHL-01* | 100% | *0.0* | 99% | JN030347.1 |

BLAST result for 16S rDNA of 8.2 *Lagria hirta* symbiot (colony)

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Uncultured Burkholderia sp. clone 3* | 100% | *0.0* | 98% | JQ061259.1 |
| *Burkholderia sp. XJ11* | 100% | *0.0* | 98% | KC522298.1 |
| *Uncultured Burkholderia sp. clone 49* | 100% | *0.0* | 98% | JQ061286.1 |
| *Burkholderia gladioli strain CACua-73* | 100% | *0.0* | 98% | HQ023278.1 |
| *Bacterium SZ6-4* | 100% | *0.0* | 98% | HQ624996.1 |

BLAST result for 16S rDNA of 9.1 *Lagria villosa* symbiot (bacteriome)

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli strain BgHL-01* | 100% | *0.0* | 97% | JX566503.1 |
| *Burkholderia sp. 2387* | 100% | *0.0* | 97% | JX174264.1 |
| *Burkholderia sp. 2348* | 100% | *0.0* | 97% | JX174225.1 |
| *Burkholderia sp. 2339* | 100% | *0.0* | 97% | JX174216.1 |
| *Burkholderia sp. 2333* | 100% | *0.0* | 97% | JX174210.1 |

BLAST result for 16S rDNA of 9.2 *Lagria villosa* symbiot (colony)

| Strain | Query cover | E-value | Ident. | Accession |
|---|---|---|---|---|
| *Burkholderia gladioli strain BgHL-01* | 100% | *0.0* | 99% | JX566503.1 |
| *Burkholderia sp. 2387* | 100% | *0.0* | 99% | JX174264.1 |
| *Burkholderia sp. 2348* | 100% | *0.0* | 99% | JX174225.1 |
| *Burkholderia sp. 2339* | 100% | *0.0* | 99% | JX174216.1 |
| *Burkholderia sp. 2333* | 100% | *0.0* | 99% | JX174210.1 |

Table 6: GenBank Accession Numbers of included species (outgroup +reference)

**GenBank Accession No.**

| SPECIES (BACTERIA) | 16S rRNA |
|---|---|
| *Burkholderia ambifaria strain JN3* | KF150332 |
| *Burkholderia cepacia strain LMG 1223* | HQ849078 |
| *Burkholderia oklahomensis strain LMG 23618* | HQ849092 |
| *Burkholderia stabilis strain LMG 14294* | HQ849103 |
| *Burkholderia tropica strain LMG 22274* | HQ849105 |
| *Burkholderia vietnamiensis strain LMG 10929* | HQ849107 |
| *Burkholderia plantarii strain NIAES 1723* | NR_037064.1 |
| *Burkholderia gladioli strain MS102* | EU053154.1 |
| *Burkholderia gladioli strain FE1201* | GU784923.1 |
| *Burkholderia gladioli strain ICMP 17866* | HQ148708.1 |
| *Burkholderia gladioli strain BSR3* | JF431409.1 |
| *Burkholderia gladioli strain BSR5* | JF431410.1 |
| *Burkholderia gladioli strain BgO1-1* | JX566502.1 |
| *Burkholderia gladioli strain BgHL-01* | JX566503.1 |
| *Burkholderia gladioli strain BgA1-1* | JX566504.1 |
| *Burkholderia gladioli strain 2002721590* | AY268163.1 |

| SPECIES (BACTERIA) | 16S rRNA |
|---|---|
| *Burkholderia gladioli strain 2002721589* | AY268164.1 |
| *Burkholderia gladioli strain 1996012911* | AY268166.1 |
| *Burkholderia gladioli strain 1993027208* | AY268167.1 |
| *Burkholderia gladioli strain CH1* | AY500139.1 |
| *Burkholderia gladioli strain MS102* | EU053154.1 |
| *Burkholderia gladioli strain CIP 105410* | NR044378.1 |
| *Burkholderia gladioli strain FE1201* | GU784923 |
| *Burkholderia gladioli pv. gladioli* | GU479033.1 |
| *Ralstonia insidiosa strain AU2944* | NR_025242.1 |

| ***SPECIES (INSECTA)*** | **18S rRNA** | **28S rRNA** |
|---|---|---|
| *Cerogria bryanti UPOLZL0011* | EF209951.1 | FJ903810.1 |
| *Adynata brevicollis UPOLZL0107* | EF209952.1 | FJ903875.1 |
| *Macrolagria robusticeps UPOLZL0025* | EF209959.1 | FJ903820.1 |
| *Tenebrio molitor* | X07801.1 | EU048308.1 |
| *Tribolium castaneum BT0053* | HM156711.1 | JX412253.1 |